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HOW TO COLLECT AND PROCESS SMALL POLYHEDRAL VIRUSES OF INSECTS

The past few years have seen increased interest in and use of microbial agents for the control of destructive forest insects. One of the most successful applications of this control method has been the use of the polyhedral virus disease of the European pine sawfly, *Neodiprion sertifer* (Geoff.). Control of this insect by its specific pathogen has been fully as satisfactory as chemical control, and less expensive.

The main purpose of this note is to show how the concentrated polyhedral suspension used in control operations is obtained. The steps described here apply to insects containing polyhedral bodies of 5 microns or smaller. Most sawfly polyhedroses (*Neodiprion sertifer, N. lecontei, N. pratti,* etc.) fall in this size range.

HOW TO OBTAIN THE POLYHEDRA

The actual pathogen, the virus rod, is embedded in a protective shell, the polyhedron. Hugh quantities of these are found in the insect immediately after death. Two general procedures are used to obtain the disease agent.

Field collection. European pine sawfly larvae, as well as most other sawflies that have died of polyhedrosis, turn black and dry on the twigs and needles of the host tree. Dying larvae have a yellowish cast, but these should not be included with the rest of the collected material until actually dead.

Select diseased colonies and individuals from infested trees. It is advisable not to include healthy insects in the collection. These healthy individuals cause difficulties in determining the concentration of the final polyhedral suspension because of the fatty and resinous material in them.

Field collection may be quicker than laboratory rearing if sufficient diseased colonies can be found. However, care must be exercised in removing not only the healthy larvae but also bits of bark, twigs, needles, and other materials that add to the difficulty of cleaning the final suspension.

Laboratory rearing. This method, while more time-consuming, is more efficient than field collecting when the disease is not prevalent or when field populations are sparse. In addition, the constant observation made possible by laboratory rearing permits placing dead larvae in the collecting vessel at the most advantageous time for efficient cleaning.

Several colonies (the exact number depending upon the amount of polyhedral suspension desired) are brought into the laboratory. Each colony is placed in a separate rearing container and is provided with host foliage thoroughly sprayed with the disease agent. After the larvae have consumed this foliage, they are then fed untreated foliage until death ensues, usually 12 to 20 days later, depending on the vigor of the insects and the rearing conditions. Crowding and poor sanitation in the rearing containers usually aid in the onset of the disease. If the insects are collected when in instars II-IV, the disease will strike when the larvae are nearly full-grown. This insures the maximum return of polyhedra because more polyhedra per larva are obtained from larger individuals.

In some circumstances it may be desirable to combine the two methods by spraying a few infested trees with the disease agent and then collecting diseased larvae. Beating the tree with a padded stick will enable the collector to obtain the sick and dead larvae quickly.

STORAGE OF DISEASED CADAVERS

There are two ways in which the collected cadavers may be kept prior to preparation of the final suspension. Both methods have their advantages and disadvantages.

Wet storage. The diseased larvae are collected in glass containers (pint or quart) half full of water. They

may be kept this way for a few months in the refrigerator at 4°C. When this method is used, the polyhedra do not tend to clump and stick together, and a more homogeneous suspension can be prepared. However, wet storage tends to cause deterioration of the polyhedra more quickly than does dry storage. The action of putrefactive bacteria on the carcasses may cause objectionable odors, but this can be overcome by processing the larvae quickly. For long-term storage, wet storage is not recommended.

Dry storage. The diseased larvae are collected in dry glass containers. For long-term storage of polyhedral material, this method is recommended. The virus material apparently loses very little virulence when stored in this manner for several years. Objectionable odors are avoided, and the necessity for a refrigerator will be eliminated because dry material can be stored at room temperature. However, preparation of the final suspension from the dried carcasses presents a more difficult problem because, as the insect dries, the polyhedra form large clumps, which must be specially treated to separate them.

In short, if the diseased material is collected for use the next year in the field, wet storage is acceptable; but if storage of the diseased material will be longer than a few months before the final suspension is prepared; dry storage is preferred.

HOW TO PREPARE THE POLYHEDRAL SUSPENSION

To prepare a polyhedral suspension from the diseased larvae, the following equipment is required: (1) a good compound microscope, (2) an electric centrifuge capable of speeds to 5,000 rpm, (3) a Waring-type blender, (4) a hemacytometer, and (5) pipettes, large beakers, and glass containers. It is necessary that the operating characteristics--particularly the range of speeds--of the centrifuge are known. Any one of several types of hemacytometers, or corpuscle-counting chambers, can be used; instruction booklets for the various types are invariably included with the particular device purchased.

From wet storage. First, mix or blend the cadavers thoroughly. Then filter out coarse debris by passing the material through a fine-mesh plastic screen. Wash the material remaining on the screen several times with a stream of water, and then squeeze it dry to obtain the maximum amount of polyhedral bodies in the filtrate.

This first filtrate must now be further cleaned of debris by differential centrifugation. The debris is re-

FLOW SHEET FOR

CENTRIFUGATION OF SMALL POLYHEDRA, SUCH AS N. SERTIFER VIRUS

Initial filtration after several washings and squeezings

Centrifuge at 500 rpm for 2 minutes

Sediment

Re-suspend in water and centrifuge at 2,000 rpm for 15 seconds

Sediment

Re-suspend in water and centrifuge at 2,000 rpm for 15 seconds

Sediment

Examine under microscope and discard if only a small number of polyhedra are seen. Repeat previous step if many polyhedra are present

Supernatant

Centrifuge at 750 rpm for 1½ minutes

Sediment

Supernatant

Supernatant

Combined supernatant

Centrifuge at 5,000 rpm for 30 minutes

Sediment

Re-suspend in water, mix well in blender

Centrifuge at 1,500 rpm for 1 min.

Sediment

Re-suspend in water and centrifuge at 2,000 rpm for 1 minute if appreciable number of polyhedra are present.

Sediment

Repeat precious step if many polyhedra are present. Discard if most polyhedra are removed.

Supernatant

Examine for polyhedra. If present in quantity repeat previous step; discard if few polyhedra are present.

Supernatant

Supernatant

FINAL CLEAN SUSPENSION

moved to make the determination of the concentration easier. However, it is not necessary to completely remove all the debris from the suspension.

It can be seen that several microscope examinations of the material (using the hemacytometer) are necessary in the process of centrifugation. It has been our practice to discard any material that has a concentration of less than 1×10^5 polyhedra per milliliter. The polyhedral bodies of *N. sertifer* virus disease are small (1.0-1.5 mu.) but can be recognized as apparently spherical bodies under the high dry objective (43x) of the compound microscope. These bodies tend to appear reddish and give a bullseye effect when the focus is changed slightly. Actual practice in observing known polyhedra is the best way to become familiar with the appearance of these bodies. Bright field, dark field, and phase-contrast illumination can be used. The type of illumination will be dictated by the equipment and experience of the individual.

From dry storage.

Preparation of the suspension from dry material requires additional steps at the beginning of the process to separate the clumped polyhedra and free them from the dried remains of the host tissues. First grind the dried larvae with a mortar and pestle with a few drops of a wetting agent such as Santomerse and sufficient water to make fluid. After thoroughly pulverizing the larvae, allow the material to stand overnight to permit softening. Mix this material completely in a blender and then proceed as with the wet-stored material.

DETERMINING THE CONCENTRATION

The concentration of the cleaned polyhedral suspension is determined by use of a blood cell counting chamber (hemacytometer) such as the Levy counter with improved double Neubauer rulings.

In practice, it is usually necessary to dilute the final suspension to 10 or even 100 times its volume with water before reading in the hemacytometer, because it is so concentrated that accurate counts of the polyhedra are difficult to make. Take several separate samples (usually four), and use the average to calculate the concentration of polyhedra in the final suspension.

The detailed steps in determining the concentration are as follows: (1) mix the suspension well in the blender, (2) take a sample (1-5 ml.) and dilute with water, (3) assemble the clean hemacytometer and place a quantity of the diluted sample under the cover slip by means of a pipette,

(4) allow the polyhedra to settle for 5 minutes, and (5) count the number of polyhedra.

For making the count, the improved Neubauer ruling has a central 1-mm. square, which is subdivided into 400 smaller squares. These 400 squares are divided into 25 groups of 16 squares. Double rulings separate the 25 units from one another. Count the number of polyhedra in each of the four corner units of 16 small squares plus a central unit of 16 small squares. In this manner the number of polyhedra in 80 small squares are determined. Repeat this process several times and calculate the average count. Then insert the calculated average in the following formula to determine the concentration of the particular sample:

$$\text{Concentration of polyhedral bodies per milliliter of sample} = \frac{(\text{ave. count})(\text{dilution})(4{,}000)}{80}\ (1{,}000)$$

$$= (\text{ave. count})(\text{dilution})(50{,}000)$$

The dilution is the ratio of the diluted volume to the original volume of the cleaned suspension. Thus, if 9 ml. of water were added to 1 ml. of suspension, the dilution factor would be 10 and the number 10 would be inserted in the formula. If 99 ml. of water were added to 1 ml. of suspension, the dilution factor would be 100, and this would be entered in the formula.

The concentration of the suspension should be determined immediately after preparation and again just before the preparation of the finished field spray.

STORAGE OF THE FINAL SUSPENSION

The suspensions of *N. sertifer* and other small polyhedra store well in water at 4°C. The pH of the suspension should be checked and the material buffered if necessary to keep the pH at approximately 7.0, because alkaline suspensions will deteriorate rapidly. Stored suspensions will keep their virulence for several years, but a gradual lessening of virulence may occur. Suspensions more than 2-3 years old should not be used in the field.

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The insect pathology section of the Station's Forest Insect Laboratory at New Haven has a quantity of *N. sertifer* polyhedral virus and will send a sample to anyone who is in-

terested in examining the suspension. Sufficient material will be sent to provide a starter culture for those who wish to use the laboratory-rearing method to build up a stock for field use.

Any additional information on the collecting and processing of small polyhedral suspensions will be gladly sent on request.

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